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Haemophilus Influenzae In Adults: An Analysis Of Incidence, Serotype, Biotype, Beta-Lactamase Production And Clinical Spectrum

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HAEMOPHILUS INFLUENZAE IN ADULTS: AN ANALYSIS OF INCIDENCE, SEROTYPE, BIOTYPE, BETA-LACTAMASE PRODUCTION AND CLINICAL SPECTRUM

BY

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I am grateful that this research was conceived and executed in a setting in which the information obtained may be used for improved patient care - the Veterans Administration Medical Center, Jackson, Mississippi.

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HAEMOPHILUS INFLUENZAE IN ADULTS: AN ANALYSIS OF INCIDENCE, SEROPTYPE, BIOTYPE, BETA-LACTAMASE PRODUCTION AND CLINICAL SPECTRUM

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A selective medium, chocolate agar with vancomycin, bacitracin, and clindamycin, was used to enhance the recovery of Haemophilus influenzae from adult patients. Cultures of the 634 respiratory specimens yielded 130 (20.5 percent) H. influenzae isolates, representing 93 patients. The serotype, biotype, and Beta-lactamase production of all isolates were determined. Of 103 distinct isolates, nine percent were serotypable and included serogroups a,b,d,and e. Although biotypes I, II, III, V, and VI were represented, 81.5 percent of the isolates were in the II/III group. Twenty-six percent of the isolates were Beta-lactamase producers and, therefore, ampicillin resistant.

The clinical characteristics of all patients were evaluated and used to categorize patients according to disease syndromes. Patients for whom H. influenzae was the primary pathogen were categorized as having an acute exacerbation, febrile tracheobronchitis or pneumonia. Colonized patients and those with mixed pneumonia with other pathogens identified were the remaining categories. When evaluated by clinical categories, nonserotypable organisms were the most common, irrespective of disease. Biotypes II and III predominated in all patient groups,

and the average Beta-lactamase production of the three groups with clinically important disease with H. influenzae as the primary pathogen was 33 percent.

It was determined that selective media was not needed for the recovery of H. influenzae in patients with clinically important disease. A Gram stain and chocolate agar plate were adequate when used by a skilled technologist. In general, selective media resulted in growth of H. influenzae in patients with no disease (i.e. colonized) when compared to chocolate agar only. However, selective media did prove beneficial when the patients had received antibiotics prior to culturing.

I. INTRODUCTION

Haemophilus influenzae is a fastidious, non-motile bacillus that requires the presence of X factor, hemin, and V factor, nicotinamide adenine dinucleotide (NAD), for growth. The typical gram stained appearance is a short, gram-negative rod (1).

Haemophilus influenzae was discovered and described by Pheiffer in 1892. It was thought to be the cause of influenza until 1933 when the influenza virus was discovered (2). At that time the interest in H. influenzae decreased. Although H. influenzae has always been an important pathogen in children, it has usually been ignored in adults. It is responsible for otitis media and epiglottitis and is the primary cause of meningitis in children (3,4). Within the past decade the role of H. influenzae as a pathogen in adults has been increasingly reported.

The research presented here investigated the incidence of H. influenzae infection in patients at the Veterans Administration Medical Center, Jackson, Mississippi. In addition, serotype, biotype, and Beta-lactamase production were determined for all isolates. Finally, patients in whom H. influenzae was isolated were evaluated for clinical syndromes and relationship to serotype, biotype, and Beta-lactamase production.

II. REVIEW OF THE LITERATURE

Media

The growth of *Haemophilus* species is not supported by most conventional media. The needed factors, X (hemin) and V (NAD) must be supplied. Both factors are present in blood cells; however, only the X factor is present in a usable form in blood agar. The blood must be heated as in chocolate agar to release the V factor and to inactivate V factor-destroying enzymes. With X and V factors added, a variety of media including Columbia, nutrient broth, nutrient broth No. 2, brain heart infusion and blood agar base No. 2 have been shown to support the growth of *H. influenzae* (see Table 1) (5). However, Chocolate agar (blood agar base with 10 percent horse or bovine blood) is the preferred medium to support the growth of *Haemophilus* (1).

Influential in this research was a study using selective media for the recovery of *H. influenzae* from specimens contaminated with upper respiratory flora. Chocolate agar containing vancomycin (CHOC-V), as well as chocolate agar containing vancomycin, bacitracin, and clindamycin (CHOC-VBC) and chocolate agar (CHOC) without antibiotics, were compared (6). Pharyngeal swabs from 852 children were suspended in broth and inoculated to each of the three types of media. Fifty-two, 254, and 589 strains of *H. influenzae* were isolated with CHOC, CHOC-V, and CHOC-VBC, respectively (see Table 2). The increased recovery is attributed to the suppression of normal respiratory flora rather than to enhancement of *H. influenzae* growth. Specifically, the growth of *H.*

TABLE 1

EFFECTS OF THE MEDIUM ON THE GROWTH AND THE X-FACTOR REQUIREMENT OF *HAEMOPHILUS* SPECIES [FROM TEBBUTT (5)]

Medium[a]	Percentages of Total Strains that Grew on Medium		
	V-dependent species	*H. influenzae*	*H. influenzae* Medium Supplemented with X-factor
Proteose Peptone	49	0	44
Proteose Peptone + thiamine	78	0	45
Columbia	93	6	97
Nutrient Broth	65	5	97
Nutrient Broth No.2	68	6	99
Brain Heart Infusion	75	7	93
Blood Agar Base No.2	95	5	98
Total Strains (N=)	40	167	127

[a] - NAD (10 mg/l) added to all media

TABLE 2

RECOVERY OF *HAEMOPHILUS INFLUENZAE* FROM PHARYNGEAL SWABS WITH THREE DIFFERENT MEDIA [FROM CHAPIN AND DOERN (6)]

No. of unique strains of *H. influenzae*	No. of subjects from whom strains were recovered		
	CHOC	CHOC-V	CHOC-VBC
0	801	609	344
1	50	232	431
2	1	11	73
3	-	-	4

TABLE 3

RELATIVE RECOVERY OF DIFFERENT BIOTYPES OF *HAEMOPHILUS INFLUENZAE* FROM PHARYNGEAL SWABS WITH THREE DIFFERENT MEDIA [FROM CHAPIN AND DOERN (6)]

	No. (%) of biotypes recovered		
H. influenzae biotype (No.)	CHOC	CHOC-V	CHOC-VBC
1 (128)	9 (7.0)	50 (39.1)	124 (96.9)
2 (129)	10 (7.8)	70 (54.3)	120 (93.0)
3 (148)	13 (8.9)	55 (37.2)	145 (98.0)
4 (76)	5 (6.6)	30 (39.5)	72 (94.7)
5 (13)	0	3 (23.1)	13 (100)
6 (85)	9 (10.6)	36 (42.4)	79 (92.9)
UD[a] (30)	6 (20.0)	10 (33.3)	26 (86.7)

[a] UD= undefined biotype

influenzae on CHOC-V and CHOC-VBC usually occurred in the first quadrant of streaking, which is where the normal flora grew heaviest on the CHOC. After isolation, all colonies grew well on CHOC. The author also noted that of the 609 unique strains, 125 (20.5 percent) were found to be sero group b. The number and frequency of biotypes of the *H. influenzae* that grew on the different media can be seen in Table 3. The relative recovery rate using CHOC-VBC as the primary isolation medium did not vary with the different biotypes. CHOC and CHOC-V had wider ranges of recovery of different biotypes (6).

The accidental introduction of pipe tobacco in a faulty batch of nutrient media that poorly supported the growth of *H. influenzae* led to interesting findings (7). Subsequent testing revealed that small amounts of tobacco and nicotine added to nutritionally poor media or to liquefied sputum would stimulate the growth of *H. influenzae*. It was concluded that the regular introduction of nicotine into bronchial secretions of heavy smokers might be sufficient to support excessive multiplication of *H. influenzae*.

Identification

Since the late 1800's numerous different ways to determine the factor requirements of *Haemophilus* have been used. Although satelliting and paper discs containing factors are used in many clinical laboratories, studies have shown that a porphyrin or sucrose test may be more accurate. Kilian's taxonomic study of *Haemophilus*, published in 1976 (2), proposed a biotyping system based on a variety of biochemical

tests. The Kilian system is still being studied, along with and compared to serotyping methods.

The phenomenon of "satelliting," as described by Grassberger in 1897 (2), was the first attempt to determine growth requirements of Haemophilus species and still provides a simple method for determining V-factor requirement. This phenomenon occurs when Haemophilus grows around an organism that can synthesize an excess of NAD, thereby supplying the V factor. Growth of Haemophilus around the organism on media without X factor was identified as a V-factor-requiring isolate. Satelliting on an X-factor-containing medium suggested that the organism could require the V factor only or both X and V factors. The use of both types of media could tentatively identify organisms as to factor requirements.

The satelliting technique is frequently used on BAP to identify gram negative coccobacilli to the genus level. This is done by spreading a lawn of the organism on the BAP and then streaking a NAD producing organism, usually Staphylococcus, over the inoculated area. However, the sole use of satelliting to determine V-factor requirements can lead to misidentification. For example, the NAD supplying organism needs to be a catalase-negative organism, such as Enterococcus or Lactobacillus, because some catalases are hemin-containing enzymes and can supply sufficient X factor to support the growth of some Haemophilus isolates (8). Therefore, the use of catalase producing organisms such

as Staphylococcus could result in the misidentification of X-and-V-requiring isolates as V-only-requiring ones (9).

The use of paper discs impregnated with NAD was introduced as a refinement of factor determination. Discs containing the X factor and both X and V factors are also in use. A nutrient agar is inoculated with a lawn of Haemophilus, discs are added, and satelliting requirements are determined.

Despite technical improvements with the disc method, misidentification may still occur for a variety of reasons. Some nutrient agars contain a sufficient amount of X factor to support growth of H. influenzae isolates around the V disc, resulting in misidentification. It has been suggested that no medium that can support the fastidious growth of Haemophilus could be completely free of X factor (1). Furthermore, the carry-over of X factor contained in the primary isolation medium on which the Haemophilus species is growing can also be a problem. This problem can be minimized by diluting the organism in broth before inoculating the medium. However, some X-and-V-factor-requiring strains can store a sufficient endogenous supply of hemin while growing on the primary isolation plate to allow growth around the V-factor disc. These X-and-V-factor-requiring isolates that satellite around the V-factor disc often do so with significantly less growth than around the XV-factor disc. Other strains grow equally around the XV-and V-factor discs, but the growth is usually sparce and may reflect additional nutrient requirements.

This X-factor carryover can cause confusion within the laboratory when two Haemophilus isolates from two different samples from the same sites are reported as having different factor requirements. A repeat of the test, using the growth from around the V-factor-containing disc, will give the proper reactions (9). However, technical problems may result, no matter what technique is employed. A study comparing the disc method with other methods correctly identified 400 H. influenzae isolates. Twenty-five others were incorrectly identified as H. parainfluenzae by the disc method. A repeat testing for the disc factor requirements grew around the XV factor disc and V-factor only disc with little visible difference (5).

The X-factor-containing nutrient agar and inoculum carryover can cause erroneous results even in carefully performed tests. Kilian (1) found that 18 percent of isolates were incorrectly identified by this method and developed a comprehensive testing battery for the reclassification of Haemophilus species. Some of the potentially differentiating biochemical tests will be examined.

In his comprehensive study (2), Kilian used a variety of biochemical tests to identify Haemophilus to the species level and to further divide the species. A prescribed testing regime was used and the composite reactions identified the organism. Some of the individual tests utilized by Kilian have also been used to identify the Haemophilus species. The porphyrin, sucrose, indole and o-nitrophenyl-Beta-O-galactopyranoside tests have been the most promising individual

tests to aid in Haemophilus identification and to differentiate between H. influenzae and H. parainfluenzae.

The porphyrin test is an accurate procedure for determining X-factor requirements. Hemin-independent organisms excrete porphobilinogens and porphyrins when supplied with delta aminolevulenic acid (ALA). The test is performed by inoculating the organism in a solution of ALA, incubating the solution and checking for red fluorescence with an ultraviolet light. X-factor independent strains will be positive for fluorescence, while X-factor-requiring strains will be negative (1). Kilian has used this test as a basis for separating H. influenzae and H. parainfluenzae (1). Other studies have confirmed the reliability of this test (5).

The sucrose test may also be helpful in identifying the species. The production of acid from sucrose is characteristic of H. parainfluenzae, while H. influenzae does not produce acid from gas. Although there have been some problems of reproducibility, this test may be of value for identification purposes (5).

The production of indole has been evaluated for characterizing species within the genus of Haemophilus. Although not all biotypes of H. influenzae are indole positive, no H. parainfluenzae isolates positive for indole have been identified. Therefore, indole production is also a key reaction in determining biotype (see Table 4). It should be noted that it is the growth of indole-positive strains of Haemophilus on agar media that produces the characteristic pungent odor (2).

A spot indole test has been promoted by some investigators as a quick, reliable method for presumptively identifying H. influenzae (10). The biotypes of H. influenzae that have been most frequently associated with serious Haemophilus infections are indole positive. In this study, 90 of 151 (60 percent) H. influenzae from respiratory sources and 67 of 72 (93 percent) H. influenzae from blood or CSF were indole positive. Although four strains of 117 (3 percent) H. parainfluenzae from respiratory sources were spot indole positive, these were false-positive results due to adjacent indole-producing non-haemophili organisms. As a result of this study, these authors defined H. influenzae isolates as nonhemolytic, indole-positive bacteria selectively isolated on horse blood agar. In contrast, another study found 80 percent of H. influenzae strains indole positive, but noted that 26 percent of V-factor-dependent species were also positive (5). Although the indole test is of interest and may have future value in predicting invasive disease, these results point out its limitations, which at present preclude its use as a test for presumptive H. influenzae identification (10).

The Beta-galactosidose activity of Haemophilus has been determined by ONPG testing (O-nitrophenyl-Beta-O-galactopyranoside). Studies have shown H. influenzae to be consistently negative for ONPG and H. parainfluenzae to be frequently positive (see Table 4) (2,5). The ONPG is a useful test in conjunction with other tests and can be helpful in

TABLE 4

KEY TO THE DIFFERENTIATION OF THE BIOTYPES OF *HAEMOPHILUS INFLUENZAE* AND *HAEMOPHILUS PARAINFLUENZAE*, *HAEMOPHILUS AEGYPTIUS* AND *HAEMOPHILUS SEGNIS* [FROM KILIAN (1)]

Species and Biotype	Indole	Urea	Ornithine decarboxylase
H. influenzae			
Biotype I	+	+	+
Biotype II	+	+	-
Biotype III	-	+	-
Biotype IV	-	+	+
Biotype V	+	-	+
Biotype VI	+	-	-
H. aegyptius	-	+	-
H. parainfluenzae			
Biotype I	-	-	+
Biotype II	-	+	+
Biotype III	-	+	-
H. segnis	-	-	-

ruling out H. influenzae, but it is not sufficiently accurate to be used alone in determining identification.

Serotype

The serological grouping of Haemophilus isolates has historically been a key factor in studying the epidemiology of disease and predicting virulence. In 1931 Dr. Pittman published her initial study (11) defining rough (R) and smooth (S) colony types of H. influenzae and also noting that S strains possessed capsules. Her study also suggested that the S strains were somewhat more virulent in laboratory animals. In addition, she also produced an antisera against these isolates that allowed her to group the colonies as type a or b. Since these initial studies, six serological types have been recognized, a through f.

These serogroups can be established by several recognized procedures, including slide agglutination, Quelling reaction, countercurrent immunoelectrophoresis (CIE), latex agglutination and antiserum agar (12). Although the slide agglutination has been most frequently used, there are questions about its accuracy. For example, in one study in which slide agglutination results were verified by a second method, usually CIE, 26 percent of the isolates were misidentified by routine slide agglutination (13). Problems with the slide agglutination test include autoagglutination by some non-typable strains and, perhaps, observer bias expecting the classical typing of pathogenic group b. The important role of type b H. influenzae has been well documented in children with epiglottis, otitis media, meningitis

and other infections. Specifically, type b has been reported to cause 95 percent of these serious infections in children. Although H. influenzae type b is the classical pathogen, the other groups have caused serious infections. Type a has been reported in a subcutaneous abscess and postpartum infection (14). Abscesses, respiratory tract infections and meningitis have been reported due to type c organisms (14,15). Bacteremia has been reported for a type d organism and meningitis, pneumonia, and bacteremia have been attributed to H. influenzae type e (16,17,18).

Although it was postulated that typable organisms were also the major cause of diseases in adults, a recent prospective study (13) found that almost two-thirds of isolates from adults with bacteremia or meningitis were non-typable. Non-typable H. influenzae is a recognized part of man's normal respiratory flora. It can be cultured from the nasopharynx in one- to two-thirds of normal adults. Continued investigations are studying all types of H. influenzae infections and many have been observed to be caused by non-typable organisms (13). Most adults with H. influenzae meningitis have an underlying infection with non-typable H. influenzae as the pathogen. The infection appears to be seeded by local extension, rather than a hematogenous route (19). Further, in a study of elderly patients with H. influenzae pneumonia, 85 percent of the isolates were found to be non-typable (20). Although there may not be an increase in the incidence of non-typable H. influenzae infection, there is an increase in the reporting of

non-typable H. influenzae as the etiological agent in serious infections.

Biotype

The haemophli have been determined to be a heterogenous population. While serology has been the established method for grouping H. influenzae, biochemical methods have been established which divide the species into biotypes. However, the biochemical reactions of haemophili have been hindered by lack of media capable of supporting growth. Thus, identification was based on few tests and this retarded the development of classification of new species and made exact identification difficult. In 1976, Kilian published his findings on a variety of biochemical tests and proposed revisions in the taxonomy (see Table 5). H. influenzae, H. haemolyticus, H. haemoglobinophilus, H. ducreyi, H. parainfluenzae, H. paraphrophilus, H. aphrophilus, H. segnis, H. parasuis, H. pleuropneumoniae, H. paragallmarum, and H. piscium were the species characterized. Within the species H. influenzae and H. parainfluenzae, further biochemical separations denoted biotypes. After the organism has been identified to the species level, the key tests for biotyping H. influenzae are indole, urease, and ornithine decarboxglase (see Table 4). Biotype I is positive for all three tests; biotype II, indole and urease; biotype III, urease; biotype IV, urease and ornithine; biotype V, indole and ornithine, biotype VI, indole.

TABLE 5 Differential Characteristics of Haemophilus species (from Kilian (2))

	ALA → porphyrins	V-factor requirement	Indole	Urease	Ornithine decarboxylase	H_2S (lead acetate)	Haemolysis	Glucose, acid	Glucose, gas	Sucrose, acid	Lactose, acid	Xylose, acid	Mannitol, acid	Deoxyribose, acid	β-Galactosidase (ONPG)	α-Glucosidase (PNPG)	α-Fucosidase	Catalase	Oxidase	Haemagglutination	CO_2 improves growth	Alkaline phosphatase	Nitrate reduction
H. influenzae																							
Biotype I	−	+	+	+	+	−	−	+	−	−	−	+	−	+	−	−	−	+	+	−	−	+	+
Biotype II	−	+	+	+	−	−	−	+	−	−	−	d	−	+	−	−	−	+	+	−	−	+	+
Biotype III	−	+	−	+	−	−	−	+	−	−	−	d	−	+	−	−	−	+	+	d	−	+	−
Biotype IV	−	+	−	+	+	d	−	+	−	−	−	+	−	+	−	−	−	d	d	−	−	+	+
Biotype V	−	+	+	−	+	−	−	+	−	−	−	+	−	+	−	−	−	+	+	−	−	+	+
H. haemolyticus	−	+	d	+	−	+	+	+	d	−	−	d	−	+	−	−	−	+	+	−	−	+	+
H. haemoglobinophilus	−	−	+	−	−	d	−	+	−	+	−	+	+	d	d	−	−	+	+	−	−	−	+
H. ducreyi	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+
H. parainfluenzae																							
Biotype I	+	+	−	−	+	+	−	+	d	+	−	−	−	−	+	−	−	d	+	−	−	+	+
Biotype II	+	+	−	+	+	+	d	+	+	+	−	−	−	−	d	−	−	d	+	−	−	+	+
Biotype III	+	+	−	+	−	+	d	+	−	+	−	−	−	−	d	−	−	+	+	−	d	+	+
H. paraphrophilus	+	+	−	−	−	+	−	+	+	+	+	−	−	+	+	d	−	−	+	−	d	+	+
H. ahrophilus	+	−	−	−	−	−	−	+	+	+	+	−	−	+	+	d	−	−	−	−	d	+	+
H. segnis	+	+	−	−	−	−	−	w	−	w	−	−	−	−	d	−	−	w	−	−	−	+	+
H. parasuis	+	+	−	−	−	d	−	+	−	+	−	−	−	+	d	d	+	+	−	−	−	+	+
H. pleuropneumoniae	+	+	−	+	−	−	+	+	−	+	−	+	+	+	+	−	−	d	d	−	−	+	+
H. paragallinarum	+	+	−	−	−	−	−	+	−	+	d	+	+	+	+	+	d	−	−	−	−	+	+
H. piscium	+	−	−	−	−	−	−	+	−	+	−	−	−	−	w	−	−	−	+	−	−	−	−

+, More than 90% of strains positive; −, more than 90% of strains negative; d, 11 to 89% of strains positive; w, weak reaction. ALA, δ-aminolaevulinic acid; ONPG, o-nitrophenylgalactopyranoside; PNPG, p-nitrophenylglucopyranoside.

Several multitest systems have been evaluated as means of more quickly and easily biotyping Haemophilus species. These test systems are frequently found in clinical laboratories to identify Enterobacteriacea. In some systems enriched inoculating broths are needed. However, many of the tests do not depend on growth of the organism, but detect the presence of constitutive enzymes. Indole production, urease, and ornithine decarboxylase and sometimes the o-nitrophenyl-Beta-D- galactopyranoside (ONPG) test are the biochemical properties evaluated to assign biotypes.

The API 10S and API 20E (Analytab Products, Incorporated, Plainview, New York) are 18 hour tests and have been shown to correlate 100 percent with conventional test systems (21,22). The Minitek system (BBL, Cockeysville, Maryland) requires overnight incubation and was found to have 97.7 percent agreement with conventional methods (23) while Micro-ID (General Diagnostics, Morris Plains, New Jersey), a four hour test, had 99 percent agreement (24). PathoTec strips (General Diagnostics, Morris Plains, New Jersey) and spot tests performed with filter paper soaked with solutions used for conventional tests have also been evaluated. The PathoTec strips agreed 100 percent with indole, 99.5 percent with urease, and 97.5 percent with ornithine. The spot biochemical tests agreed 99 percent with indole, 100 percent with urease, and 96 percent with ornithine when compared to conventional methods. Both methods can be read in less than 15 minutes. They have

been proposed as a more economical method, since only the tests used in biotyping need to be performed (25).

All of the systems studied were accurate and reproducable. The variety of systems evaluated suggests that one system is usually already available in most clinical laboratories and, therefore, affords a means for quickly and accurately biotyping Haemophilus.

Methods for further classifying the haemophili have been explored. The relationship between IgA proteases and serotypes has been studied (26). It was noted that type 1 protease was produced by serotypes a,b,d and f and that type 2 protease was produced by serotypes c and e. H. influenzae primarily infects the mucosal surfaces where defense is mediated by secretory IgA. Production by some bacteria of an enzyme to cleave IgA may be a factor in pathogencity. Sub typing non-typable H. influenzae, based on outer-membrane proteins (OMP) (27), and encapsulated H. influenzae with polyribosylribitolphosphate (PRP) (28) has also been utilized to characterize these organisms.

Biotyping is proposed as the most reliable epidemiological marker for H. influenzae. Although capsular typing has been an important tool, it is not as reliable because of the heterogeneity of the organism and the failures of reproducability in serotyping (22). Because of its wide battery of tests, and its reproducibility, biotyping is being examined as an epidemiological tool (31). With the emergence of several rapid methods for biotyping Haemophilus, most institutions will be able to

easily determine biotypes that will undoubtedly be valuable markers for epidemilogical studies.

Correlation of Serotype and Biotype

Correlations between biotypes and serological groups have been made. Studies comparing biotype and serotype have found most typable strains to be biotype 1, although a few non-typable H. influenzae are biotype 1 (13,29). In a study by Kamme of 393 isolates, 89 percent of capsulated strains were biotype I or IV, and 95 percent of non-capsulated strains were biotypes II, III, or V (30). This seems to indicate that the ability to produce capsular polysaccharide in most strains is associated with the production of urease and ornithine decarboxylase and that non-encapsulated strains produce only one of the enzymes.

The relationship between age, site of infection, serotype and biotype of H. influenzae has been studied by Mehtar et al. (22). H. influenzae isolated from children were predominately from cerebrospinal fluid (CSF), blood, and conjuctiva; whereas, the adult isolates were primarily respiratory. The children's isolates were usually biotypes I and III and predominately serotype b. In Mehtar's study, approximately two-thirds of H. influenzae isolated from the over forty age group were non encapsulated. Lack of or loss of immunity in these patients might be a contributing factor. Also, long term colonization of chronic bronchitis could cause the loss or gain of a capsule. Age may be a factor in biotype and serology grouping.

For encapsulated organisms, the serological groups were distributed somewhat equally among group a, b, c and the combination d, e, f. Biotype II was most common in adults, whether serotypable or nonserotypable. Another study reported biotype I predominately from children less than one year of age. Biotypes II and III were found in the 1-5 and over 20 age group (31).

The site of infection correlates with a somewhat predictable biotype. Thus, adult respiratory isolates were more likely biotype II, and childrens' H. influenzae isolates associated with meningitis were more likely biotype I. It might be extrapolated that the age predilection might revert back to the fact that certain age groups are more prone to certain types of infections.

Serotype/Biotype vs. Antibiotic Resistance

Several studies have drawn similar conclusions regarding the relationship of biotype and encapsulated strains to antibiotic resistance. Biotype 1 encapsulated strains have the highest incidence of ampicillin resistance due to Beta-lactamase production. In one study, 13 of 15 ampicillin-resistant organisms were serotype b and biotype I and II (29). Albritton's study (32) examining 100 blood isolates found 8 percent of the resistant organisms to be biotype I and found no resistant organisms among the other biotypes. Of the 500 non-blood isolates tested, 34 in biotype I/II and one non-I/II biotype were found to be resistant to antibiotics. Kamme (30) also found a prevalence of biotype I Beta-lactamase positive strains among blood/CSF

isolates, as well as nasopharynx/throat isolates. In addition to this biotype correlation, all of the Beta-lactamase positive isolates were also serotypable. In contrast, Long and associates (3), in analyzing H. influenzae isolates exclusively from children, noted that there appeared to be less antibiotic resistance among serotypable isolates than among non-typable and less resistance among biotype I than among other biotypes. It is not known if the exclusive population of Long's study, as opposed to the other studies that included both pediatric and adult isolates, may explain the differing results. In summary, however, the majority of the reports in the literature concluded that typable isolates are more frequently Beta-lactamase positive and that the predominant biotype among antibiotic resistant organisms is I.

Serotype/Biotype vs. Site of Infection

Relationships between biotype or serotype and site of isolation have been noted. The most common correlations are as follows: biotype I, blood and CSF; biotypes II and III, respiratory, eye, ear; other biotypes, a variety of sites, the majority of which are respiratory. In addition, almost all serotypes isolated from blood or CSF are type b. Isolates found in otitis media, conjunctivitis, and respiratory isolates are also predominately type b. The other serotypes do not appear to be associated with particular sites of isolation, being noted equally at all sites.

Albritton, et al. (32) correlated biotypes and site of infection among both bacteremic and nonbacteremic patients. Of 100 isolates from

bacteremic patients, 34 percent with meningitis, 10 percent with epiglottitis, 4 percent with pneumonia, 5 percent with septic arthritis, 8 percent with cellulitis, and 4 percent from other sources were biotype I organisms. The percentages in biotype II were 12 percent meningitis, 5 percent epiglottitis, 5 percent pneumonia, 3 percent cellulitis, and 3 percent from other sources. Biotypes III and IV had 4 and 3 isolates, respectively, and comprised the remaining isolates. The 100 isolates from nonbacteremic patients were mostly biotype II. Sources of biotype II isolates included 19 percent eye, 20 percent sputum, 6 percent lung, 2 percent pharynx, 1 percent ear, and 1 percent other sources. Other notable percentages were sputum isolates that were 21 percent biotype I and 10 percent biotype III. The remaining 20 percent was scattered among the biotypes.

In Oberhofer and Back's study (31) on 464 H. influenzae isolates, impressive figures validated previously presented correlations. Of 227 eye isolates, 130 were biotype II and 86, biotype III. Thirty-eight of 70 nasopharynx isolates were biotype II. Of 63 sputa, 22 isolates each were identified as bioytpes II and III. Thirty-four of 36 blood and 14 of 15 CSF isolates were biotype I. Of seven tracheal aspirates, 4 were biotype II. The 21 ear isolates were divided into four predominate biotypes. Four ear isolates were biotype I, 6 isolates were biotype II, 4 isolates were biotype III, and 5 isolates were biotype VI.

Studies correlating serotype and site of infection have emphasized the importance of serotype b (29). In a study of adults with a variety

of clinical syndromes, 46 (53 percent) of 87 isolates were type b; 4 (5 percent) were of another type; and 24 (28 percent) were non-typable (13). Serotype b organisms have also been noted to be the predominate isolates from children with invasive disease, otitis media and conjunctivitis. In addition, non-pathogenic respiratory isolates were also more common among pathogenic respiratory isolates of H. influenzae, 58 percent of typable isolates were serotype b, while the other 42 percent involved serogroups a,e, and f (3). Thus, serotype b organisms are the predominate serogroup noted in infections of both children and adults.

Serotype/Biotype vs. Virulence

Biotype and serotype have been associated with virulence. The polysaccharide capsule of type b Haemophilus has been determined to be a major factor in its pathogenicity. Although the capsules are immunologically and chemically the same, the existance of noncapsular virulence has also been suggested (3). Wallace et al. (13) suggested that nontypable H. influenzae were the major cause of invasive disease in adults. Biotypes I and II have been recovered principally from patients with systemic disease. The other biotypes have been associated with localized infection and as commensal respiratory flora (29). It has been suggested that some biotypes, especially biotype I, may be indicators of virulence factor(s), in addition to or along with capsular antigens (13). Long et al. (3) concluded that virulence was associated with biotype I, with or without encapsulation.

Beta-lactamase and Antimicrobial Sensitivity

Prior to the 1970's, it was generally agreed that Haemophilus was universally susceptible to ampicillin and would remain so. In 1972, the Center for Disease Control (CDC) received a H. influenzae isolate from a United States Army laboratory in Germany (33). The organism showed genetic characteristics of resistance to ampicillin and produced a Beta-lactamase. This organism was the first of a series of resistant H. influenzae isolates reported from throughout the United States and eventually most parts of the world. There is still no explanation as to how these stains became resistant by the same plasmid mediated mechanism in different parts of the world.

Beta-lactamase production is the primary mechanism of ampicillin resistance in Haemophilus. The Beta-lactam antibiotics must have an intact Beta-lactam ring in order to interfere with bacterial cell wall synthesis, thus causing the cell to lyse. Most resistant H. influenzae produce Beta-lactamase, an enzyme that hydrolyzes the Beta-lactam ring. The resulting component, pencilloic acid, has no antibacterial activity. Thus far, H. influenzae isolates have been shown to produce only the TEM Beta-lactamase enzyme (33).

The genes responsible for drug resistance are primarily found on plasmids, circular pieces of extra chromosomal DNA. A plasmid gene coded for antimicrobial resistance is also known as an R factor. This resistance can be transferred from cell to cell and can cross species lines. The transfer of genetic material requires cell-to-cell contact

and is called conjugation. Haemophilus resistance is believed to have occurred by transposon, the nonreciprocal insertion of material deleted from one chromosome into another. A species containing the TEM or type IIIa Beta-lactamase gene can be inserted into an indigenous plasmid in H. influenzae (33,34). Ampicillin-resistant strains of H. influenzae that do not produce Beta-lactamase have been recovered, but the mechanisms mediating this resistance are not known (35,40). These are frequently non-typable respiratory isolates from patients receiving long-term treatment (33).

There are three basic methods for determining an organism's ability to produce Beta-lactamase (33,36). The iodometric (starch-iodine) test and the acidometric test rely on a pH change resulting from the penecilloic acid produced when the Beta-lactamase breaks down the Beta-lactam ring of penicillin. The chromogenic cephalosporin test uses the substrate nitrocefin, which changes color when acted upon by Beta-lactamase due to changes in the molecular configuration. All methods are reliable.

The percentage of resistant Haemophilus changes from community to community, but nationwide the percentages have increased in recent years. The rates can vary by patient population, but overall resistance is believed to be between 18-22 percent (33).

A greater incidence of Beta-lactamase production was noted among encapsulated strains than among non-capsulated strains in a Swedish study (30). The increased isolation of capsulated Beta-lactamase

producers from the upper respiratory tract may be due to isolation of strains from selected patients. It was theorized that this information could indicate that capsulated strains have a greater ability to acquire plasmids conferring resistance than non-capsulated strains have. The 283 non-capsulated isolates have members in all of the possible biotypes (I-VI). However, when the non-capsulated isolates were examined for Beta-lactamase production, groups I and IV were not represented. It was suggested that this could indicate that non-capsulated strains producing both urease and ornithine decarboxylase are unable to acquire plasmids conferring ampicillin resistance.

Studies comparing antimicrobial susceptibility, Beta-lactamase production and biotypes have been done. Granato et al. (29) found that 13 of 15 ampicillin-resistant Beta-lactamase producers belonged to biotype I and the majority (85 percent) were type b. Another group (41) reported two of the 78 (2.6 percent) biotypable strains of H. influenzae ampicillin-resistant due to Beta-lactamase production both belonging to biotype II.

The literature cites cases in which both Beta-lactamase positive and negative H. influenzae have been isolated in children and adults. In two cases (37) Beta-lactamase negative organisms were identified in the CSF and Beta-lactamase positive organisms in the blood. A study by Stewardson-Krieger et al. (38) revealed two morphologically distinct colonies from a CSF. Both colony types were confirmed to be H. influenzae type b, but one was Beta-lactamase positive and one was

Beta-lactamase negative. Another case (39) demonstrated only one colony type from the blood, but discrepancies in Beta-lactamase production and disc diffusion susceptibility testing identified both a Beta-lactamase positive and a Beta-lactamase negative strain. These cases serve as a reminder to clinical microbiologists that a single colony from a culture is not necessarily the only phenotype and that bacteriological subgroups can occur. This also emphasizes the need for clinical correlation of *in vivo* response.

These studies emphasize that the routine use of ampicillin without the determination of Beta-lactamase production may result in clinical failures. In addition, other antibiotics resistant to Beta-lactamase inactivation or combination containing Beta-lactamase inhibitors should be evaluated both *in vitro* and *in vivo*.

Haemophilus Infections in Adults

Haemophilus influenzae is most frequently thought to be a cause of disease in children. However, serious *H. influenzae* infections in adults are being reported with increasing frequency (13,42). Although most of the cases reported are pulmonary in origin, *H. influenzae* has been found to be the etiological agent in a wide spectrum of clinical infections. For example, a recent 18 month prospective study of *H. influenzae* in hospitalized adults from September, 1976, through February, 1978, yielded 110 clinical isolates (43). Of these cases, 10 isolates were from patients with bronchitis; 25, pneumonia; and 65, respiratory tract colonization. No extra-pulmonary infections due to

Haemophilus were identified. However, in an eight-year retrospective review, these same authors identified 16 cases of serious extrapulmonary H. influenzae infections in adults. These cases included meningitis, pericarditis, epiglottitis, cellulitis, osteomyelitis, endometritis, urinary tract infection, orbital cellulitis, primary peritonitis, mesenteric lymphadenitis and aortic graft infection. Of interest was the fact that over three-fourths of the patients grew H. influenzae along with other pathogens, most frequently Staphylococcus aureus.

Despite the varied clinical infections caused by H. influenzae, respiratory tract infections remain the most frequent disease seen in adults. For example, Berk and co-workers performed trans-tracheal aspirates (TTA) in 104 adult patients and identified 14 (13 percent) with H. influenzae pneumonia (20). The majority of these patients had underlying chronic lung disease and had acquired their pneumonia in the community, the nursing home and hospital setting. A second prospective study by Gleckman et al. noted H. influenzae to be the second most common cause of community acquired pneumonia in adult patients needing hospitalization (44).

The clinical features of bronchopulmonary infections due to H. influenzae are similar to those in disease caused by Streptococcus pneumoniae (45). However, expectorated sputum with apple-green coloration is suggestive of H. influenzae infection. The results of sputum gram stain and culture are confirmatory. Patients reported as having H. influenzae bronchopulmonary infections have the following

characteristics: 60 percent or greater are older than 50 years of age; 30 percent have a history of alcoholism; 30-60 percent have chronic lung disease; and 25 percent have the onset of their pneumonia about two weeks after a presumed viral respiratory tract infection (45). Cavitary pneumonia due to H. influenzae is most often seen in alcoholic patients (46).

As previously discussed, a wide variety of extrapulmonary infections have been noted with H. influenzae. Although frequently described in children, cases of epiglottitis are also encountered in adults (47,48). Septicemia in association with epiglottitis is frequently noted and, unless recognized early, is often fatal.

Pericarditis associated with H. influenzae pneumonia and bacteremia has been reported (49). It should be noted that the two cases described were effectively treated with antibiotics alone. Spontaneous bacterial peritonitis associated with chronic ascites has also been reported (50). In these cases, bacteremia from a respiratory site is the presumed pathogenesis.

Cellulitis of neck as a complication of pharyngitis has been caused by H. influenzae (51). In addition, cellulitis at sites distant from the respiratory tract have also been reported in adults (52). In these cases, bacteremia from a respiratory source is the presumed precipitating factor. The clinical picture of cellulitis in adults may mimick that seen in children; however, the onset may be slower and the cellulitis may appear erythematous rather than the characteristic

blue-purple color seen in children. Meningitis has also been noted in both immunocompromised and presumably normal adults (53,54).

In addition to the syndromes noted above, mediastinitis, biliary tract infections, bursitis, septic arthritis and other focal sites of infection have been noted with H. influenzae in adult patients (53,55,56,57). Finally, overwhelming post-splenectomy sepsis has also been described in an adult (49). It is of interest that this patient had received prior immunization with pneumococcal vaccine.

III. MATERIALS AND METHODS

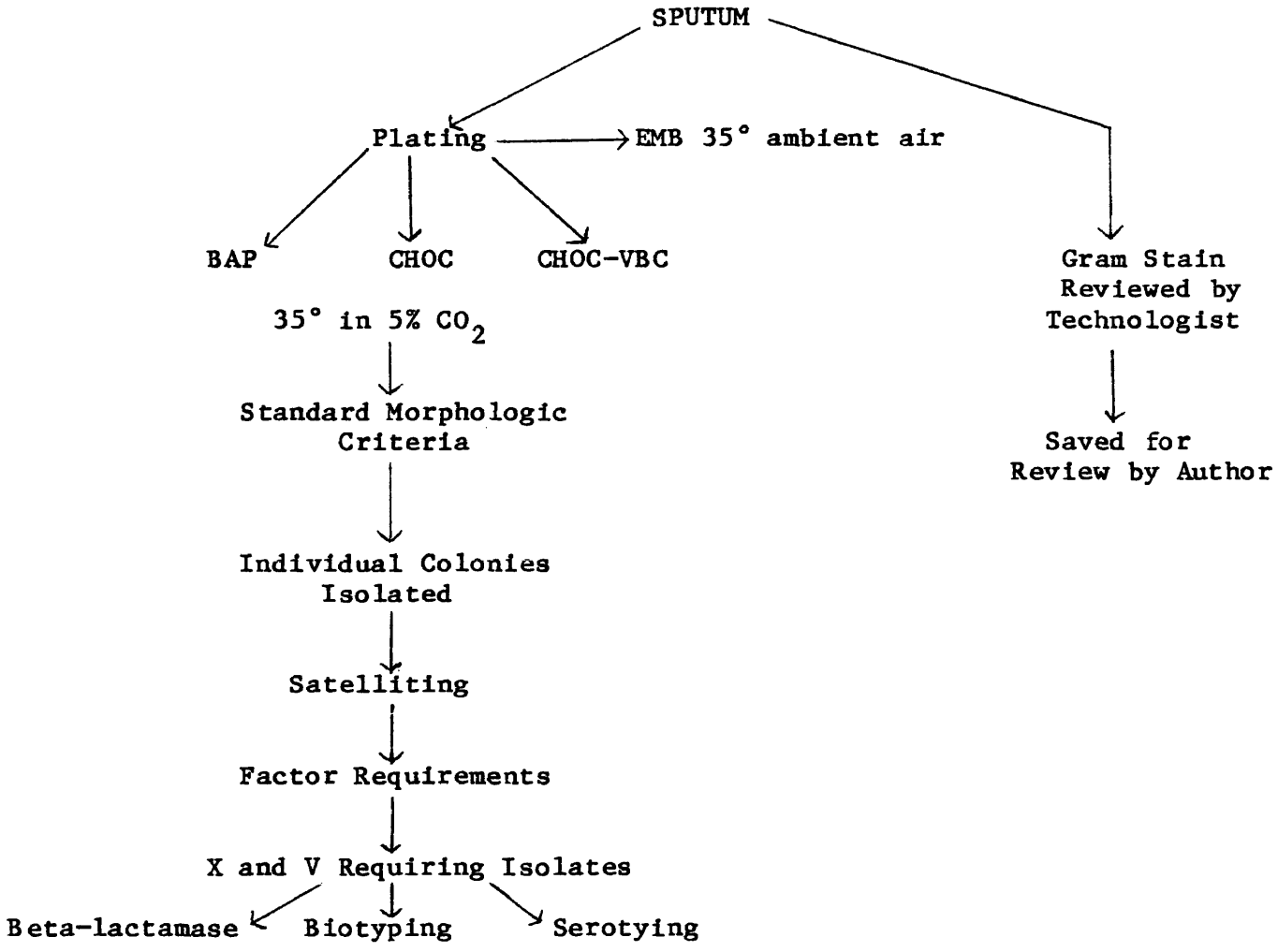
Specimen Protocol

During a three and one-half month period (December 28, 1983, to April 10, 1984), all routine sputum cultures received in the clinical laboratory at the Veterans Administration Medical Center, Jackson, Mississippi, were plated on a chocolate agar plate [CHOC] (GC Agar Base, Hemoglobin and IsoVitalex, BBL, Cockeysville, Maryland), a sheep blood agar plate [BAP] (Trypticose Soy Agar, BBL, Cockeysville, Maryland with 5 percent defibrinated sheep blood, Scott Laboratories, Fiskeville, Rhode Island) and an eosine methylene blue agar plate [EMB] (BBL, Cockeysville, Maryland). In addition, a chocolate plate containing vancomycin, bacitracin, and colistin [CHOC-VBC] (Advanced Scientific, Incorporated, Chalmette, Louisiana) was also inoculated, using quadrant streaking. The CHOC, BAP, and CHOC-VBC were incubated overnight in 5 percent CO_2 at 35°C. The EMB was incubated overnight in room air at 35°C. Initially the CHOC-VBC was read at 48 hours as suggested by other authors (6). However, after 150 consecutive sputums had been examined, it was determined that the amount of growth did not change with additional incubation, only the size of the colonies. Therefore, all plates were subsequently evaluated at 24 hours of incubation only (see Table 6 for Protocol Summary).

A smear was also made at the time the sputum was plated and was stained by Hucker's Gram stain procedure, read by the bench

TABLE 6

SPECIMEN PROTOCOL



technologist, and saved. The smear was re-examined to classify the quality of the sputum sample and quantity of Haemophilus-type rods. The smears were graded in categories based on the number of segmented neutrophils and white blood cells (see Table 7).

The typical short gram negative rod (GNR) was the criteria for judging quantity of "Haemophilus" in the smear. The presence of typical gram-negative rods were scaled 0-4+, depending upon the number per oil power field.

In addition to evaluation by the medical technologist, all routine plates were also evaluated by the author for growth of colonies exhibiting morphological characteristics of H. influenzae. On CHOC, colonies of H. influenzae are typically grayish, semiopaque, smooth, flat convex and entire (1). The amount of growth was quantified according to the following scale: light, growth in the initially inoculated area; moderate, growth in the second and third quadrants; and heavy, growth in the fourth quadrant.

All colonies with typical Haemophilus appearance were isolated and then screened for X and V requirements by satelliting on BAP, utilizing S. aureus as a V source. This testing was done by streaking the suspicious colony types onto a BAP and then placing a streak of known S. aureus perpendicular to the "Haemophilus" streaks. The test is considered positive for satelliting if colonies grow in the area immediately surrounding the Staphylococcus streak. This provides a

TABLE 7

GRADING SPUTUM SMEARS [FROM WASHINGTON ET AL. (59)]
CELLS (NO./FIELD)[a]

Group	Leukocytes	Squamous epithelial cells
6	<25	<25
5	>25	<10
4	>25	10-25
3	>25	>25
2	10-25	>25
1	<10	>25

a X100 magnification

preliminary identification of Haemophilus species on which further biochemical tests are performed.

Colonies found to be positive for satelliting were then tested for factor requirements. This was accomplished by inoculating isolated colonies into 5 ml of trypticase soy broth [TSB] (BBL, Cockeysville, Maryland) with a swab. The swab was twisted in the broth to release the colonies and to dilute any factor carry-over from the chocolate plate. The swab was gently rung out to eliminate excess solution. A plate of heart infusion agar [HIA] (Difco, Detroit, Michigan) was inoculated in four planes. Discs (Difco, Detroit, Michigan) impregnated with factor X,V and XV were positioned on the HIA with flamed forceps, forming an equidistant triangle approximately 15 mm apart. The inoculated HIA was incubated overnight at 35° with 5 percent CO_2, and growth factor requirements were determined by the location of growth.

The organisms requiring both X and V factors were considered to be Haemophilus influenzae. After confirmation of morphology by Gram stain, isolated colonies were frozen at -20°C in a 38 by 12.5 mm tube containing 1 ml of defibrinated sheep blood (Scott Laboratories, Fiskeville, Rhode Island). Beta-lactamase testing, biotyping, and serotyping were also performed on these isolates.

Serotype

For serologic testing, specific antisera (Difco, Detroit, Michigan) were used. Control organisms of serogroups a-f were kindly provided by Dr. R. Facklam (CDC, Atlanta, Georgia). A drop of

polyvalent antisera was placed on a partitioned glass slide and a control drop of saline was placed on a corresponding portion. Applicator sticks were used to mix the colony first with the saline solution and then, using a clean stick and more organisms, with the antisera. The slide was rocked for one minute. Aggultination in the polyvalent sera was followed by typing with each specific serogroup a-f, using antisera and saline control as described previously. The organism was assigned to the serogroup in whose antisera it agglutinated.

Biotype

Biotype specific reactions were determined, utilizing the Micro-ID (General Diagnositics, Morris Plains, New Jersey) enteric identification system after the technique of Kilian (1). The tray was inoculated as specified in the manufacturer's instructions for identification of enterics. The inoculated tray was incubated in room air at 35°C for four hours. This rapid test detects the presence of specific enzymes and/or metabolic products and does not depend upon growth. The nitrate reductase, indole, ornithine decarboxylase and urea results were determined. A biotype category could then be assigned using Kilian's criteria (see Table 5).

Beta-lactamase

A rapid acidometric test for Beta-lactamase used commercially prepared Beta-ase Tubes (Microbiological Specialities, Phoenix, Arizona). Five drops of water were added to the tubes containing 15 gm

penicillin, 5 gm sodium chloride, 0.018 gm phenol red, 1.5 gm trisodium citric acid, and 0.3 gm trisodium phosphate. The tube was tapped gently to dissolve the powder. A small loop or applicator stick was used to inoculate the colony into the solution. Staphylococcus aureus ATCC 29213 was used for a positive control and S. aureus ATCC 25923 was used for a negative control. The tubes were left at room temperature and examined for a color change from red to yellow within fifteen minutes.

Patients' Chart Review/Clinical Correlation

Each patient's chart was reviewed for specific clinical and laboratory criteria for inclusion in a defined data base. Those parameters reviewed and entered for each patient's data base included sputum Gram stain and culture results, age, chest x-ray results, admission diagnosis, and discharge diagnosis. The presence or absence of the following were also evaluated: symptoms related to the respiratory tract (i.e. cough, dyspnea, sputum production, hemoptysis, etc.), other medical illnesses, fever, and leukocytosis.

Patients were then categorized into five clinical groups, utilizing the following definitions:

(1) Colonized - absence of fever, leukocytosis, or pulmonary infiltrate on x-ray; in other words, individuals with no evidence of acute pulmonary disease.

(2) Acute exacerbation of chronic bronchitis - an increase in respiratory symptoms (cough, sputum production, etc.) without fever, leukocytosis or abnormal chest x-ray.

(3) Febrile tracheobronchitis - an increase in cough and sputum production, associated with fever and/or leukocytosis. The chest x-ray revealed no pulmonary infiltrates.

(4) H. influenzae pneumonia - a pulmonary infiltrate on chest x-ray associated with pulmonary symptoms, fever and leukocytosis. H. influenzae was the only or predominate organism on sputum culture.

(5) Mixed pneumonia - a pulmonary infiltrate on chest x-ray, pulmonary symptoms, fever and leukocytosis. H. influenzae was isolated in association with other potential respiratory pathogens and was not the predominant organism.

Each individual chart was reviewed by the author and an experienced Infectious Diseases Clinician in establishing the patient's clinical grouping.

IV. RESULTS

Frequency of Isolation

Six hundred and thirty-four respiratory specimens were examined. Of these, 259 organisms (41 percent) were isolated that required V factor only and were presumed to be *H. parainfluenzae.* One hundred thirty specimens (20.5 percent) grew *H. influenzae* requiring both X and V factors. In 11 specimens, *H. influenzae* and *H. parainfluenzae* isolates were simultaneously identified. The 130 specimens yielding *H. influenzae* isolates were obtained from 93 patients. Sixty-six patients had only one specimen yielding *H. influenzae*. Twenty-seven patients had two or more specimens with positive cultures. Five isolates from four patients were not viable after freezing, leaving 125 *H. influenzae* culture isolates that were further characterized.

Isolates from the 27 patients with two or more cultures positive for *H. influenzae* were further evaluated to determine whether these represented the same or different organisms. Each patient's isolate was compared for biotype, serogroup reaction, and Beta-lactamase production. Cultures from the same patient with the same results in all three testings were considered to be the same isolate. Four cultures' isolates differed by biotype and Beta-lactamase production; four, by biotype; and four, by Beta-lactamase production. These isolates were considered different organisms in subsequent analysis.

As a result of these tests, 103 different *H. influenzae* isolates, obtained from 89 different patients, were available for further study. The data presented will be examined by isolates or specimen isolates as

needed to present the most accurate reflection of percentages for the category being evaluated.

Media

The growth of H. influenzae on CHOC and CHOC-VBC was compared. Of cultures positive for H. influenzae that received both platings, approximately 60 percent of the isolates that grew on CHOC-VBC grew on CHOC. Cultures growing H. influenzae heavily were comparable on both media; however, discrepancies were found in lesser quantities of growth (see Table 8).

Serotype

Of the 103 different isolates, nine (9 percent) were typable with H. influenzae antisera. Two isolates were typable in serogroup a; two, in serogroup b; one, in serogroup d; and 4, in serogroup e. The growth of typable isolates on CHOC and CHOC-VBC is summarized in Table 9. The only discrepancy was "usual flora" reported from a CHOC that grew H. influenzae moderately on CHOC-VBC. Three of the nine isolates (33.3 percent) were Beta-lactamase producers. H. influenzae biotypes II (3 isolates), III (4 isolates) and VI (2 isolates) were represented in this group.

Biotype

The Micro I.D. was used to determine Haemophilus biotypes. Of the 103 isolates, two were nonviable at the time of biotyping. Thus, of the 101 remaining isolates, the following biotypes were determined: I-- 5

TABLE 8

HAEMOPHILUS ISOLATES RECEIVING BOTH PLATINGS

	CHOC	CHOC-VBC
Heavy	54	52
Moderate	9	31
Light	1	25
No Growth	40	-
Others	5[a]	1[b]

a - Haemophilus species requiring V only isolated
b - CHOC-VBC overgrown with Proteus species; moderate growth isolated from CHOC

TABLE 9

TYPABLE *HAEMOPHILUS INFLUENZAE* ISOLATES

Amount of Growth	CHOC	CHOC-VBC
Heavy	6	4
Moderate	0	2
Light	0	3
No *H. influenzae* isolated	4	0
No CHOC-VBC	-	1

TABLE 10

BETA-LACTAMASE PRODUCING *HAEMOPHILUS INFLUENZAE*

Amount of Growth	CHOC	CHOC-VBC
Heavy	22	12
Moderate	1	5
Light	0	2
No *H. influenzae* isolated	4	0
No CHOC-VBC plated	-	8

isolates; II -- 50 isolates; III -- 36 isolates; V — 2 isolates; and VI -- 8 isolates.

Beta-lactamase

The rapid acidometric test for Beta-lactamase production demonstrated 27 producers (26.2 percent) representing 19 patients (21.3 percent) and 20 different isolates. It should be noted that in five patients, Beta-lactamase negative isolates were also cultured, in addition to the Beta-lactamase positive organisms.

Of the 27 Beta-lactamase producers, 22 grew heavily on CHOC and 12 grew heavily on CHOC-VBC. Eight specimens grew moderately and two grew lightly on CHOC-VBC. One specimen grew moderately on CHOC. H. influenzae was not isolated from four specimens plated on CHOC (see Table 10).

Only three Beta-lactamase positive patients (15.8 percent) had isolates that were serotypable: one each in groups b, c, and e. The Beta-lactamase producers showed the following distribution for biotypes: I (1 isolate); II (9 isolates); III (6 isolates); V (1 isolate); and VI (3 isolates).

Gram Stain

The stained sputum smears were examined by the laboratory technologist and then by the researchers. The smears were categorized by quality and quantity as previously described. The category and number of isolates were grouped as follows: Group 1 -- 2 isolates; Group

2 — 5 isolates; Group 3 — 34 isolates; Group 4 -- 9 isolates; Group 5 -- 35 isolates; Group 6 -- 3 isolates; and gram stained smears were not found for four isolates. The quanitity of Haemophilus was categorized as follows: <1+ growth -- 7 isolates; 1+ growth — 11 isolates; 2+ growth -- 23 isolates; 3+ growth -- 21 isolates; 4+ growth -- 26 isolates; and 4 isolates for which the gram stained smear was not found.

Patients' Chart Review

The hospital charts of the 93 patients from whom H. influenzae was recovered were reviewed. Using several key characteristics, the patients' medical problems were divided into clinical syndromes by a clinician. The factors examined included the following: age, white blood cell count, fever, sputum production, hemoptysis, presence or absence of an infiltrate, Gram stain and culture results, admitting diagnosis, reason for primary admission, discharge diagnosis, and antibiotics received. The categories and the number of patients assigned to the group included patients colonized with H. influenzae (45), patients colonized with H. influenzae and associated with acute exacerbation (AE) (10), febrile tracheobronchitis (FTB) (11), pneumonia (18), mixed pneumonia with other pathogen identified (8). In addition, one patient had sinusitis, meningitis and bacteremia from H. influenzae.

V. DISCUSSION

Introduction

It was determined from this study that 20 percent of the patients of the Jackson, Mississippi, Veterans Administration Medical Center who had respiratory specimens submitted for culture during this period grew H. influenzae. The majority of these were non-typable and were predominately biotypes II and III. Approximately 26 percent of the isolates were Beta- lactamase producers. The Gram stain was recognized as an excellent predictor of H. influenzae isolation and CHOC agar was determined adequate for detecting the growth of H. influenzae from patients with infections caused by H. influenzae.

Factor Requirements

Paper disc impregnated with X, V and XV were used for factor determination. Several technical details were noted during the study. Although the manufacturer's suggestion of 30mm between the disc was used at first, it was decided that the growth patterns were more easily read if using approximately 15 mm between the disc. It was also noted that isolates requiring both X and V factors can faintly grow around the V disc. As confirmed by others (5), this is probably due to factor carry-over from the original medium, an inescapable trace of X factor in the medium on which the satelliting was done or the fact that some isolates can carry over the factors. These isolates usually grow much heavier

around the XV disc than around the V only disc. It has been reported (9) that repeating the growth factor requirements, using the growth from around the V disc, is helpful in giving clearer results on repeat testing.

It was also observed that the NAD of the V disc could disseminate into the agar further than the X only disc. As a result, some XV-requiring isolates grew at the point between the X disc and V disc where both factors were present. This occurred closer to the X-factor disc. The growth, however, was only on the side of the X disc closest to the V disc and not circumferential around the X disc.

As a final technical point, the growth around the disc can be difficult to interpret. During this study it was found that sometimes the growth was more easily seen when the back of the petri dish was observed.

Media

Chapin and Doern (6) suggested that use of CHOC-VBC to suppress growth of upper respiratory tract (URI) microbial flora would aid in recovery of H. influenzae from specimens contaminated with URI flora.

This author's study found that comparable growth of H. influenzae occurred on CHOC and CHOC-VBC. The CHOC-VBC detected light growth of the organism, while "usual flora" was noted on the CHOC plate. This latter group was, in most cases, assigned to the group of colonized patients. Of the patients with H. influenzae infections, three out of four of the isolates that grew on CHOC-VBC but not on CHOC were from

patients who had had antibiotic therapy prior to culturing. Thus, CHOC-VBC may have improved yields in patients given antibiotics prior to admission.

The CHOC-VBC cost from $.40 to $1.48, depending upon the source. Therefore, it was determined that experienced technologists were able to routinely identify signficant growth of typical H. influenzae morphology, utilizing only the CHOC, and that the CHOC-VBC would be useful for detecting signficant isolates only when antibiotics were administered before culturing.

Serotype

The literature review alluded to the difficulties in serologically testing of H. influenzae. Difficulty in interpreting serological test results were also observed during this author's study despite carefully following the manufacturer's instructions. Some isolates reacted strongly in the polyvalent sera; however, specific group test results were less strong and not easily interpreted. Several trained technologists observed the questionable reactions and the collective best judgment was used.

The actual isolates that were serotypable were few (9 percent). This emphasizes the already reported importance of non-typable isolates in adults (19,20,60,61). Adult respiratory H. influenzae isolates, pathogenic or non-pathogenic, are most frequently non-typable. In children, however, Long et al. (3) report that almost all invasive disease is caused by encapsulated isolates.

Of the nine different isolates identified, four were serogroup e. Serogroups a and b each had two isolates. Group c had one isolate. Each of these serogroups has been associated with respiratory infections (3,15,18,29). Studies have also shown that respiratory H. influenzae isolates are predominately biotypes II and III. Similar results were noted in our study with 84 of 103 (81.5 percent) isolates in biotypes II and III.

Prior studies have shown that encapsulated strains are most frequently biotypes I or VI and that a high percentage of non-typable strains are biotypes II, III, or V. In this author's study, none of the five biotype I isolates were serotypable, but two of the six biotype VI isolates were typable: one was type a and one type e. Although no biotype V isolates were isolated, biotypes II and III were 75 percent non-typable. The seven that were typable were distributed as follows: biotype II, one isolate type a and two isolates type b; biotype III, one isolate type c and 3 isolates type e.

Biotype

Eighty-two percent of biotypable isolates were group II or III. These have been the predominant biotypes suggested for respiratory isolates (30). Further, in this author's study of the three patient groups with signficant H. influenzae infection, 83 percent were biotypes II or III. This is in conflict with the reports in children that invasive disease is most frequently associated with biotype I (3).

Beta-lactamase

An increase of H. influenzae resistance to ampicillin by Beta-lactamase production has been noted by Dr. Thornsberry at the Center for Disease Control (33). In this author's study, 21.3 percent of H. influenzae patients at the Veterans Administration Medical Center were Beta-lactamase positive. This concurs with McCarthy's prediction of a current rate of 20 percent Beta-lactamase producing isolates (9).

Gram Stain

The smear of the sputum is an excellent tool for predicting Haemophilus recovery. Two factors are important in the analyses of gram stained material, including the quality of sputum as defined by polymorphonuclear leukocytes and the amount of organisms visible. Groups IV, V and VI represent the better quality sputum samples. Forty-seven of the 88 isolates (53 percent) with available smear reports were in this category. Forty-seven isolates (53 percent) had significant evidence, 3+ or 4+ of Haemophilus on the smear. When both factors were examined for each isolate 28 (32 percent) had significant quantity of Haemophilus-type organisms and a good quality of sputum. Thirty-nine other isolates had either the quality or quantity factor in the higher range.

The gram stained smears were examined by disease categories. Of the 42 colonized isolates with smears, 7 (17 percent) had both good quality and significant quantity, and 19 other isolates (45 percent) rated high in one category. The AE had 5 of 10 isolates (50 percent)

with both factors and an additional 3 (30 percent) with one factor. FTB had 4 of 11 isolates (36 percent) with both factors signficant and 6 isolates (55 percent) with one factor. The pneumoniae were 7 of 18 isolates (39 percent) with 8 other isolates (44 percent); the mixed pneumoniae were 5 of 8 (63 percent) and the remaining 3 had one significant factor.

The quality of the sputa of the different disease categories showed the highest percentages in the non-colonized. The percentages of patients with Group IV, V, or VI grade sputum were as follows: colonized, 38 percent; AE, 70 percent; FTB, 64 percent; pneumoniae, 65 percent; mixed pneumoniae, 75 percent. Thus, the quality of the sputum of patients with a significant H. influenzae isolate was better overall than the quality of sputum of patients who were colonized.

The quantity of Haemophilus-like organisms seen in the smear was compared to the quantity of growth of H. influenzae. All of the smears with 3+ or 4+ organisms grew moderately to heavily on either the CHOC or CHOC-VBC, except one. The single isolate with 3+ organisms on the smear was a Group I quality sputum from a colonized patient.

The gram stained smear is one of the most basic tests available in a laboratory. In this study it has proven to be an invaluable indicator of H. influenzae infection from good quality specimens.

Patients' Chart Review

Evaluation of Clinical Syndromes: The patients' charts were reviewed and the previously described categories were assigned. The

divisions identified patients with H. influenzae as the most likely cause of infection, as opposed to patients whose respiratory tract had become colonized.

The largest group (48 percent) was the "colonized." Although some of the patients had a heavy growth of H. influenzae, it was not believed that the organism was causing a problem for the patients but was simply a part of the patient's commensal flora.

The "mixed pneumonia with other pathogens identified" group included patients from whom H. influenzae was recovered along with other bacteria. Three patients also had Streptococcus pneumonia identified. Staphylococcus aureus was found in two patients. Escherichia coli and Citrobacter diversus were identified in one patient each. The eighth patient was an alcoholic with well-documented aspiration pneumonia from whom H. influenzae was recovered only from the CHOC-VBC.

The single uncategorized patient had H. influenzae recovered in a blood culture. The patient also had sinusitis and meningitis associated with squamous cell carcinoma of the maxillary sinus.

The remaining groups - associated with acute exacerbation, febrile tracheobronchitis and pneumonia - represent the patients for whom H. influenzae was the primary pathogen. These combined categories represent 42 percent of the patients.

Media: As the results were evaluated in light of the significance of the isolation of H. influenzae, the statistics took on additional meaning. The growth of H. influenzae on the different media was

compared (see Table 11). Eighty-six percent of the patients with respiratory isolates had at least one positive culture with both media inoculated. All positive cultures found on the CHOC were also found on the CHOC-VBC. Thirty-six percent of the isolates from colonized patients were positive on the CHOC-VBC but negative on the CHOC. However, in the AE, FTB, and pneumonia groups all except four (12.5 percent) grew on CHOC as well as CHOC-VBC. Essentially anyone with the diagnosis of AE, FTB, or pneumonia had 88 percent concordant cultures (28 of 32 cultures). Three of the four patients had antibiotics prior to the sputum culture.

Serotype: The majority of the isolates did not type; however, the ones that did type were primarily in the group of colonized patients (66 percent). A single isolate (11 percent) in the FTB, pneumonia, and mixed pneumonia each were serotypable. The serogroups were b, e and e, respectively.

Biotype: The biotypes, Beta-lactamase production, and serotyping of the patients in the different clinical groups were examined. Ten patients had two isolate types that were tested. As expected for respiratory sites, biotypes II and III were predominate in all categories. They ranged from 49.5 percent in the II category to 36 percent in the III category and were 85 percent of the total. Biotypes I, V, and VI were identified in much smaller quantities. They represented 5 percent, 2 percent, and 7.9 percent respectively.

TABLE 11

PATIENTS WITH BOTH MEDIA PLANTED

	CHOC		VBC
	+	-	+
Colonized	26	15	41
AE	7	2	9
FTB	8	2	10
Pneumoniae	13	0	13
Mixed pneumoniae	3	3	6
Total number of patients	79		79

Indole has been suggested as an indicator of virulence (10). Of all the clinical syndromes, 58.2 percent of the isolates produced indole. For the three categories of patients for whom H. influenzae was the identified cause of disease, the following percentages of indole positive isolates were noted: AE, 66.7; FTB, 50; and pneumonia, 83.3. Their combined percentage was 66.7. These statistics concur with other studies indicating that indole may indeed be a predictor of virulence in H. influenzae.

Beta-lactamase: The Beta-lactamase production for the clinical groups was calculated. Five isolates were lost in freezing and were not included. Eleven percent of the colonized patients produced Beta-lactamase. Patients associated with acute exacerbation had 30 percent Beta-lactamase producers. Febrile tracheobronchitis compromised 36 percent Beta-lactamase positive isolates, while 33 percent of pneumonia patients were positive. Only 11 percent of the mixed pneumonia were positive, and the single blood isolate was not positive for Beta-lactamase production.

The three clinical groups identified with H. influenzae as the primary pathogen were highest in Beta-lactamase produce. The percentage of these combined groups was 33 percent.

VI. SUMMARY

The data presented indicate that H. influenzae is an important respiratory pathogen in adults at the Veterans Administration Medical Center, Jackson, Mississippi. Non-serotypable organisms are the predominant cause of disease in the patients and the overwhelming majority are biotypes II and III. Ampicillin-resistant organisms, as mediated by Beta-lactamase production, occurred in 26 percent of all isolates. When isolates causing disease were analyzed, 33 percent were Beta-lactamase producers. The presence of disease correlated well with the Gram stain of purulent sputum showing greater than 2+ organisms and at least moderate growth on chocolate agar. A selective medium containing antibiotics was not necessary for the isolation of clinically significant isolates; rather, only chocolate media is necessary. This results in a cost savings to the laboratory.

The data derived have been helpful in the practical operation of the laboratory. Chocolate agar is routinely used in the isolation of potential respiratory pathogens in addition to the previously routine EMB and BAP. Beta-lactamase analysis is now routinely done on all respiratory H. influenzae isolates having a moderate or greater growth on four quadrant streaking. Prior to this research project only blood and CSF isolates were studied.

Clinically, this study has defined the spectrum of disease caused by H. influenzae and the importance of Beta-lactamase producing

non-serotypable strains. Ampicillin can no longer be considered empirical treatment in patients with serious disease caused by H. influenzae. Trimethoprim sulfamethoxasole, chloramphenicol, cephalosproins resistant to Beta-lactamase (i.e. cefamandole) or antibiotics containing Beta-lactamase inhibitors (i.e. Augmentin) should be utilized.

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